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14. ABSTRACT <p>Development of effective resuscitation agents for blood-loss replacement in trauma or surgery is extremely important. We synthesized 15 peptides (test peptides) that were designed on the basis that the heme group in myoglobin (Mb) and hemoglobin (Hb) is sandwiched between helices E and F. The abilities of the test peptides and 6 control peptides to form stable complexes with heme were investigated. None of the controls bound heme. However, each of the test peptides was able to form 1:1 coordinate complexes with heme, which were stable to manipulation and behaved as a single molecular species. The reduced peptide-heme complexes bound oxygen and gave visible spectra that were typical of oxygenated heme-proteins. The heme-peptides gave hyperbolic oxygen-saturation curves, but showed slightly different P50 values. They were administered into mice, either as emulsions in adjuvant or intravenously in PBS. Injection with adjuvant stimulated anti-peptide antibody responses, which bound to the correlate protein (Mb or Hb). But the peptides were non-immunogenic when administered intravenously in PBS.</p>					
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## FINAL REPORT

GRANT NUMBER: N00014-00-1-0452

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### OBJECTIVE

The objective of this research is to develop resuscitation agents that would be effective under conditions of anticipated emergency use (hemorrhagic shock).

### APPROACH

Development of effective resuscitation agents for blood-loss replacement in trauma or surgery is extremely important despite substantial improvements in screening methods of blood from human donors. Our approach to this problem was to design and synthesize peptides that mimic the natural environment of the heme group in myoglobin (Mb) and in the  $\alpha$ - and  $\beta$ -subunits of human adult hemoglobin (Hb). The designs were based on the fact that the heme group in the aforementioned proteins is sandwiched between helices E and F. We synthesized 15 test peptides and determined their ability to form stable complexes. Each of the test peptides was able to bind one mole of heme per mole of peptide forming a stable peptide-heme complex. We also made 6 control peptides and found that they did not bind heme. We then determined by CD measurements whether, as the peptide bound heme, it would undergo a major change in conformation reflected in an increase in helical content.

The heme-peptides were each administered into mice, either as emulsions in adjuvant (both for injections and boosters) or intravenously as solutions in phosphate-buffered saline. Antibody (Ab) responses, monitored up to 14 weeks after the first administration.

### ACCOMPLISHMENTS

#### 1. Design of the Peptides

The structures of Hb and Mb served as the basis for the design of the peptides. The peptides were designed and synthesized to mimic the environment of the heme group in Mb and in the  $\alpha$ - and  $\beta$ -chains of Hb. The strategy was based on the fact that the heme group in these three protein chains is sandwiched between helices E and F. Therefore, the peptides comprised essential parts of the segment E-F.

In Mb and the two Hb subunits, the heme group inserts in the bend between the E and F helices. The penta- and hexa-coordinate ligands of the heme iron are occupied by histidine F8 (the 8th position in helix F) and, via a water bridge, by histidine E7. A peptide corresponding to the segment E-F will carry these two essential histidine residues as well as several of the other contacts. Such a segment would therefore be expected to form

complexes with the heme group. The following three synthetic design strategies were adopted:

(a) We synthesized 3 peptides corresponding to the following sequence positions in Mb and the two Hb subunits:

Group A Peptides (Segment E7—F9):

		No. Residues	Mol Weight
A1	HGVTVLTA LGAILKKKGHHEAELKPLAQSHAG	32	3,312.87
A2	HGKKVADALTN AVAHVDDMPNALSALSDLHAG	32	3,239.61
A3	HGKKVLGAFSDGLAHL DNLKGT FATLSELH <u>CG</u>	32	3,337.80
A4	HGKKVLGAFSDGLAHL DNLKGT FATLSELH <u>AG</u>	32	3,305.70

In A4, Cys  $\beta$ 93 was replaced by Ala to avoid dimerization.

(b) In the 3-D structure of Mb and Hb, the corner of the EF bend is far from the heme group and does not make any contribution to the heme cavity. As it approaches the bend, the polypeptide chain goes out of the environment of the heme group at residue E14. Then the chain folds back so that residue F1 comes close (6.7 Å) to residue E14. We therefore made a peptide corresponding to the segment E4 to E14, bridged the gap E14 to F1 by a glycine spacer and continued the synthesis from F1 through FG5:

Group B Peptides (E4—E14)  $\leftarrow$  6.7Å  $\rightarrow$  (F1—FG5):

B5	LKKHGVTVLTA (G) LKPLAQSHATKHKIG	27	2,834.40
B6	VKGHGKKVADA (G) LSALSDLHAHKLRVG	27	2,765.21
B7	VKAHGKKVLGA (G) FATLSELH <u>CD</u> KLHVG	27	2,816.32
B8	VKAHGKKVLGA (G) FATLSELH <u>AD</u> KLHVG	27	2,784.26

B8 was an analogue of B7 with Cys93 replaced by Ala.

(c) In another set, we bridged the gap (6.8 Å) between F1 and E18 (instead of E14):

Group C Peptides (E4—E18)  $\leftarrow$  6.8Å  $\rightarrow$  (F1—FG5):

C9	LKKHGVTVLTA LGAI (G) LKPLAQSHATKHKIG	31	3,188.85
C10	VKGHGKKVADAL TNA (G) LSALSDLHAHKLRVG	31	3,164.66
C11	VKAHGKKVLGAFSDG (G) FATLSELH <u>CD</u> KLHVG	31	3,222.71
C12	VKAHGKKVLGAFSDG (G) FATLSELH <u>AD</u> KLHVG	31	3,190.65

C12 was an analogue of C11 with Cys-93 replaced by Ala.

(d) The fourth set of peptides was designed by taking into account the fact that the five residues F9 through FG4 are also away from the sphere of influence of the heme group. Furthermore they make a turn so that residue F8 comes to within 4.9Å from residue FG5. We therefore synthesized a fourth set in which F8 was directly linked to FG5 via a spacer as follows:

Group D Peptides (E4—E14)  $\leftarrow$  6.7Å  $\rightarrow$  (F1—F8) 4.9Å (FG5):

D13	LKKHGVTVLTA (G) LKPLAQSH--IG	22	2,268.73
D14	VKGHGKKVADA (G) LSALSDLH--VG	22	2,159.47
D15	VKAHGKKVLGA (G) FATLSELH--VG	22	2,219.62

### Control Peptides

The following peptides were also synthesized and used as controls

Cont1	HFKSFHSFSVSGETVFEVTEA	21
Cont2	IKAHEVPSIKSASPKSLVITEPTSKSA	27
Cont3	IPQEKSVITPGEPPIPHQPEVIKSL LHQIVSA	31
Cont4	LSHIPGYLIFGLLFRFGHTVAL	22
Cont5	LLHVPGNFL LGLLFHFGHKVAL	22
Cont6	LLHVPGNFL LGLLFSFGHKVAF	22

### 1. Binding of heme to the peptides

We developed a titration procedure to determine the ability of the synthetic peptides to bind ferriheme (Atassi and Childress, 2005a). These titrations showed that each peptide bound at equivalence one mole of heme per mole of peptide. None of the control peptides bound heme (Atassi and Childress, 2005a).

### 2. Oxygen-binding properties of the heme-peptide complexes

2.1. Spectral measurements. Spectral measurements were carried out in the range 450-700 nm before and after reduction of the heme-peptide solutions from the ferri form to the ferrous form. After reduction, the heme-peptide complexes gave spectra that were typical of the oxygenated forms of heme proteins (Atassi and Childress, 2005a). The spectra of the oxygenated heme-peptide complexes showed sharp maxima which, depending on the peptide, were at 436-547 nm and 570-582 nm (Atassi and Childress, 2005a).

2.2. Oxygen-saturation curves. The peptide-heme complexes gave hyperbolic oxygen-saturation curves (Atassi and Childress, 2005a). The complexes however showed some differences in their  $P_{50}$  values. The  $P_{50}$  values ranged from 3.8 mmHg for the heme-peptide B7 complex to 13.7 mmHg for the heme-peptide D13 complex. This can be compared with the values for human hemoglobin ( $P_{50}$  = 34.0 mmHg) and myoglobin ( $P_{50}$  = 5.5 mmHg) done under the same conditions.

### 3. Mouse response to iv administration of the heme-peptides in PBS

The heme peptides were each administered intravenously (iv) into mice as solutions in PBS. The test sera from these mice were analyzed for the presence of Ab binding to the heme-peptide as well as to intact Mb and Hb (Atassi and Childress, 2005b). Of the mice that received the A group peptides sera from only one mouse that received peptide A1 had Abs that bound to the peptide and to the parent protein (Mb). One mouse that received peptide A3 gave somewhat lower level Abs against the peptide, but these Abs did not bind to the parent whole protein (Hb). None of the other mice that received Groups A, B, C or D peptides mounted any Ab responses to the peptide or to the parent protein.

### 4. Mouse responses to injections of heme-peptides in adjuvant

To check whether the lack of immunogenicity observed in iv administration of the heme-peptides was intrinsic in the structure of the peptides or was due to the protocol of the administration, we injected each of these compounds into mice under conditions designed to elicit anti-peptide Ab

responses (Atassi and Childress, 2005a). The mice immunized with Group A peptides mounted Ab responses against heme-peptides A1 (5 of 5), A2 (3 of 5) and A3 (5 of 5). Abs of 3 mice that received peptide A1-heme reacted with the correlate protein (Mb). Abs from the 3 mice that responded to A2-heme, bound to the immunizing peptide but not to the correlate protein (Hb). Abs of the 5 mice that responded to A3-heme bound to the immunizing peptide and to the correlate protein (Hb). None of the Abs against B5-heme or B6-heme bound to the correlate protein. No Ab responses were obtained in any of the 5 mice that received B7-heme. Of the 5 mice that mounted Ab responses against C9-heme, 3 gave Abs that bound slightly to the correlate protein (Mb). Four of the mice that responded to C10-heme had high, medium or low levels of Ab binding to the correlate protein (Hb). Three of the 4 mice that responded to C12-heme had high and low Ab levels that bound to the correlate protein. The mice that received Group D heme-peptides usually mounted low Ab responses to the immunizing heme-peptide and only one, immunized with D13-heme, gave Abs that unexpectedly reacted at low levels with both the correlate (Mb) as well as the unrelated protein (Hb).

### CONCLUSIONS

It is concluded from the present findings that: (1) peptide constructs can be made that bind one mole of heme per mole of peptide; (2) as expected, oxygen-binding to the heme-peptides did not show cooperativity and gave hyperbolic oxygen saturation curves; (3) a mixture of equimolar quantities of an  $\alpha$ - and a  $\beta$ -peptide of a given design showed a  $P_{50}$  oxygen saturation value that was higher than the average of the  $P_{50}$  values of the individual peptides, indicating the presence in solution of some interactions between the  $\alpha$ - and  $\beta$ -peptides; (4) the heme-peptides injected with adjuvant stimulated Ab responses against the immunizing peptide, which in most cases bound to the correlate protein (Mb or Hb); (5) these heme-peptides were non-immunogenic when administered in PBS intravenously.

### SIGNIFICANCE

These studies have provided strong evidence that peptides of relatively small size can be designed that would form stable complexes with heme and that these heme-peptide complexes bind oxygen. The lack of immunogenicity of these heme-peptide complexes when administered intravenously in PBS is encouraging and indicates that their use in vivo would be without immunological detriment to the recipient.

PATENTS: None

### PUBLICATIONS (ATTACHED)

The following papers, which are accepted for publication, are attached:

Atassi' M.Z. and Childress, C. (2005a) Oxygen-binding Heme Complexes of Peptides Designed to Mimic the Heme Environment of Myoglobin and Hemoglobin. *Protein J.*, 24, in press.

Atassi' M.Z. and Childress, C. (2005b) Immunogenicity of Heme Complexes of Peptides Designed to Mimic the Heme Environment of Myoglobin and Hemoglobin. *Protein J.*, 24, in press.